

## University of Groningen

### Directed evolution of an enantioselective lipase

Liebeton, Klaus; Zonta, Albin; Schimossek, Klaus; Nardini, Marco; Lang, Dietmar; Dijkstra, Bauke W.; Reetz, Manfred T.; Jaeger, Karl-Erich

*Published in:*  
Chemistry & Biology

*DOI:*  
[10.1016/S1074-5521\(00\)00015-6](https://doi.org/10.1016/S1074-5521(00)00015-6)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2000

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Liebeton, K., Zonta, A., Schimossek, K., Nardini, M., Lang, D., Dijkstra, B. W., Reetz, M. T., & Jaeger, K-E. (2000). Directed evolution of an enantioselective lipase. *Chemistry & Biology*, 7(9), 709-718.  
[https://doi.org/10.1016/S1074-5521\(00\)00015-6](https://doi.org/10.1016/S1074-5521(00)00015-6)

#### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

#### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

# Directed evolution of an enantioselective lipase

Klaus Liebeton<sup>1,2</sup>, Albin Zonta<sup>2</sup>, Klaus Schimossek<sup>2</sup>, Marco Nardini<sup>3</sup>, Dietmar Lang<sup>3</sup>, Bauke W Dijkstra<sup>3</sup>, Manfred T Reetz<sup>2</sup> and Karl-Erich Jaeger<sup>1</sup>

**Background:** The biocatalytic production of enantiopure compounds is of steadily increasing importance to the chemical and biotechnological industry. In most cases, however, it is impossible to identify an enzyme that possesses the desired enantioselectivity. Therefore, there is a strong need to create by molecular biological methods novel enzymes which display high enantioselectivity.

**Results:** A bacterial lipase from *Pseudomonas aeruginosa* (PAL) was evolved to catalyze with high enantioselectivity the hydrolysis of the chiral model substrate 2-methyldecanoic acid *p*-nitrophenyl ester. Successive rounds of random mutagenesis by ep-PCR and saturation mutagenesis resulted in an increase in enantioselectivity from  $E=1.1$  for the wild-type enzyme to  $E=25.8$  for the best variant which carried five amino acid substitutions. The recently solved three-dimensional structure of PAL allowed us to analyze the structural consequences of these substitutions.

**Conclusions:** A highly enantioselective lipase was created by increasing the flexibility of distinct loops of the enzyme. Our results demonstrate that enantioselective enzymes can be created by directed evolution, thereby opening up a large area of novel applications in biotechnology.

<sup>1</sup>Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität, D-44780 Bochum, Germany

<sup>2</sup>Max-Planck-Institut für Kohlenforschung, D-45470 Mülheim/Ruhr, Germany

<sup>3</sup>Laboratory of Biophysical Chemistry, Rijksuniversiteit Groningen, NL-9747 AG Groningen, The Netherlands

Correspondence: Manfred T Reetz and Karl-Erich Jaeger

E-mail: reetz@mpi-muelheim.mpg.de

E-mail: karl-erich.jaeger@ruhr-uni-bochum.de

**Keywords:** Directed evolution; Enantioselectivity; Random mutagenesis; Saturation mutagenesis; *Pseudomonas aeruginosa* lipase

Received: 10 April 2000

Revision requested: 15 May 2000

Revisions received: 30 May 2000

Accepted: 30 May 2000

Published: 1 August 2000

**Chemistry & Biology** 2000, 7:709–718

1074-5521/00/\$ – see front matter

© 2000 Elsevier Science Ltd. All rights reserved.

PII: S 1074-5521(00)00015-6

## Introduction

Enantiomerically pure compounds are of rapidly increasing importance to the chemical industry being used as pharmaceuticals, agrochemicals, flavors and fragrances [1–4]. This trend has several reasons: (i) biological activity is often restricted to one of the two enantiomers; (ii) the ‘wrong’ enantiomer may exert undesirable effects; and (iii) registration of new drugs requires the production of the desired enantiomer in pure form. The total sales volume for enantiopure pharmaceuticals alone was estimated to reach 100 billion \$US for the year 2000 [4]. Although not all of these can be expected to be prepared synthetically, the need to develop efficient methods to obtain chiral compounds enantioselectively constitutes a major challenge in current organic chemistry. In this world-wide endeavor, various approaches have been described. Although economically and ecologically not always optimal, classical methods based on chemical or chromatographic separation of enantiomers are still widely used. Alternatively, compounds in the chiral pool of optically active natural products can be modified chemically. Theoretically, the most efficient strategy is based on asymmetric transformations using either chiral transition metal catalysts [5]

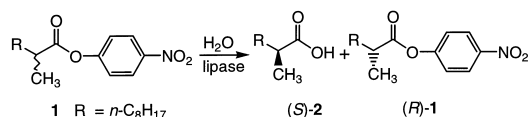
or enzymes [6]. However, for many organic substrates, catalysts displaying acceptable degrees of enantioselectivity are not available. Therefore, any new approach which allows the creation of a highly enantioselective catalyst for a reaction of choice is of great importance.

Directed evolution is a powerful technique that has been used to improve various properties of enzymes [7]. Accordingly, enzyme genes are subjected to iterative cycles of random mutagenesis and/or recombination, expressed in a suitable host system and subsequently screened or selected for improved variants. A number of non-recombinative and recombinative mutagenesis methods have been developed (summarized in [8]). These methods have to be combined with appropriate screening or selection methods which make possible the unequivocal identification of mutants having the improved properties. Successfully improved enzyme properties include activity, substrate specificity, thermostability and activity in organic solvents (for reviews, see [7,9]). Evolution of enantioselective enzymes was hampered so far by an apparent lack of appropriate screening or selection methods. Only recently, methods were described which allow for high-throughput screening

of enantioselectivity [10–14]. Moreover, since enantioselectivity is a difficult parameter to deal with, it was not certain whether directed evolution would actually constitute a viable method.

The first decision to be made when attempting to apply directed evolution in the creation of enantioselective biocatalysts is the choice of the enzyme. Among the enzymes used in organic chemistry, lipases play the most important role [15–17], mainly for the following reasons: (i) they catalyze the hydrolysis of esters in aqueous medium as well as esterification and transesterification in organic solvents, (ii) they accept a wide range of different substrates, and (iii) they do not require cofactors. Lipases used for biotransformations mainly originate from fungi and bacteria, with those derived from the genus *Pseudomonas* being particularly important [15,18]. They were studied in detail with respect to regulation of transcription, overexpression and mechanism of secretion [19].

Recently, we have used the extracellular lipase from *Pseudomonas aeruginosa* (PAL) to demonstrate the principle of creating an enantioselective enzyme by directed evolution [10]. In doing so, a substrate was chosen for which PAL shows essentially no enantioselectivity in the hydrolysis reaction, namely the kinetic resolution of 2-methyldecanoic acid *p*-nitrophenyl ester. In just four rounds of mutagenesis using ep-PCR, the selectivity factor *E* was increased from 1.1 to 11.3 (corresponding to an enantioselectivity of *ee* = 81% at 25% conversion) in favor of the (*S*)-configured acid. In each case, a library of about 1000–5200 mutants was created by employing a low mutagenesis rate.



In this publication, we describe a comprehensive directed evolution approach to evolve PAL for the enantioselective hydrolysis of the model substrate. The result of this effort includes the idea of combining random and directed mutagenesis which lead to a highly enantioselective lipase variant in which five amino acid substitutions have occurred. The structural consequences of these mutations are discussed on the basis of the PAL three-dimensional (3D) structure which was recently solved [20].

## Results and discussion

### Directed evolution of PAL enantioselectivity by ep-PCR

A total of six generations of PAL variants were produced by ep-PCR and library sizes of 1000–7000 clones were screened leading to the identification of one variant with an increase in enantioselectivity corresponding to an *E*-value of 13.5 (far right in Figure 1). The ep-PCR method used to produce these six generations was experimentally adjusted to give a mutation rate of 1–2 base substitutions

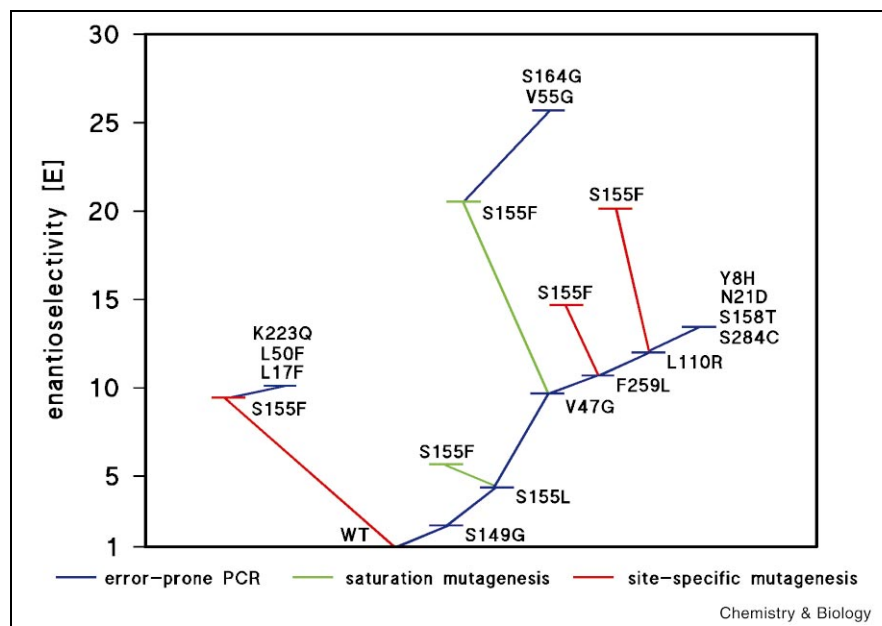
per lipase gene corresponding to a maximum of two amino acid substitutions per lipase molecule. Due to the degeneracy of the genetic code, only about 2/3 of all base substitutions result in an amino acid substitution. Therefore, at the mutation rate used here, about one amino acid substitution per lipase molecule was obtained. Assuming that just one amino acid is changed per lipase molecule and each amino acid could be substituted for by the remaining 19 others, the theoretical number of different PAL variants to be produced is 5415 [10]. The relatively small size of this library allows one to screen every single variant individually. If the mutation rate was to be increased so as to produce two instead of one amino acid substitutions per lipase molecule, a library of at least 14.6 million different variants would result. Such a huge number cannot be screened for improved enantioselectivity by existing methods nor are appropriate selection methods presently available.

The low mutation rate used here prevented the introduction of all of the remaining 19 amino acids at each position in PAL. Substitution of one nucleotide per codon by any other nucleotide leads to nine new codons encoding 4–7 different amino acids depending on the codon under investigation. Analysis of the codon usage in the lipase gene *lipA* revealed that 1852 different variants could theoretically be produced by changing one nucleotide per lipase gene. This calculation is based on the assumption that transitions and transversions occur at the same frequency. A DNA sequence analysis of 12 different variants produced by ep-PCR confirmed that in principle both kinds of substitutions were obtained. However, 22 transitions outnumbered nine transversions by a factor of 2.4. The transversions A → C, C → G and G → C were not detected at all. A biased distribution of the different kinds of base substitutions produced by ep-PCR has been described before [21,22] and might be overcome by increasing the mutation rate in the PCR reaction and simultaneously reducing the cycle number because the accumulation of mutations is cycle-dependent [23]. Consequently, this bias in the distribution of base substitutions further lowered the diversity of the PAL library bringing the number of variants to a value between 1852 and 610 with the latter number being produced if only transitions were to occur. Nevertheless, our results obtained with PAL allow the conclusion that screening of a small number of variants already allows one to identify biocatalysts with improved enantioselectivity.

### Saturation mutagenesis enhances the effectiveness of directed evolution

As shown above, the power of random mutagenesis has to do with the fact that amino acid positions in PAL responsible for improved enantioselectivity can be identified without any knowledge of the 3D structure of the enzyme. However, mainly due to the restricted diversity in the

**Figure 1.** Evolution of *P. aeruginosa* lipase enantioselectivity. The combination of different mutagenesis methods (error-prone PCR and site-specific saturation mutagenesis) to evolve an enantioselective lipase is shown. The substitution S155F was introduced into wild-type lipase and certain variants by site-specific mutagenesis, as indicated. The enantioselectivities of wild-type lipase and variants which served to parent the next generations are represented as  $E$ -values. The amino acid substitutions found in these variants are indicated.



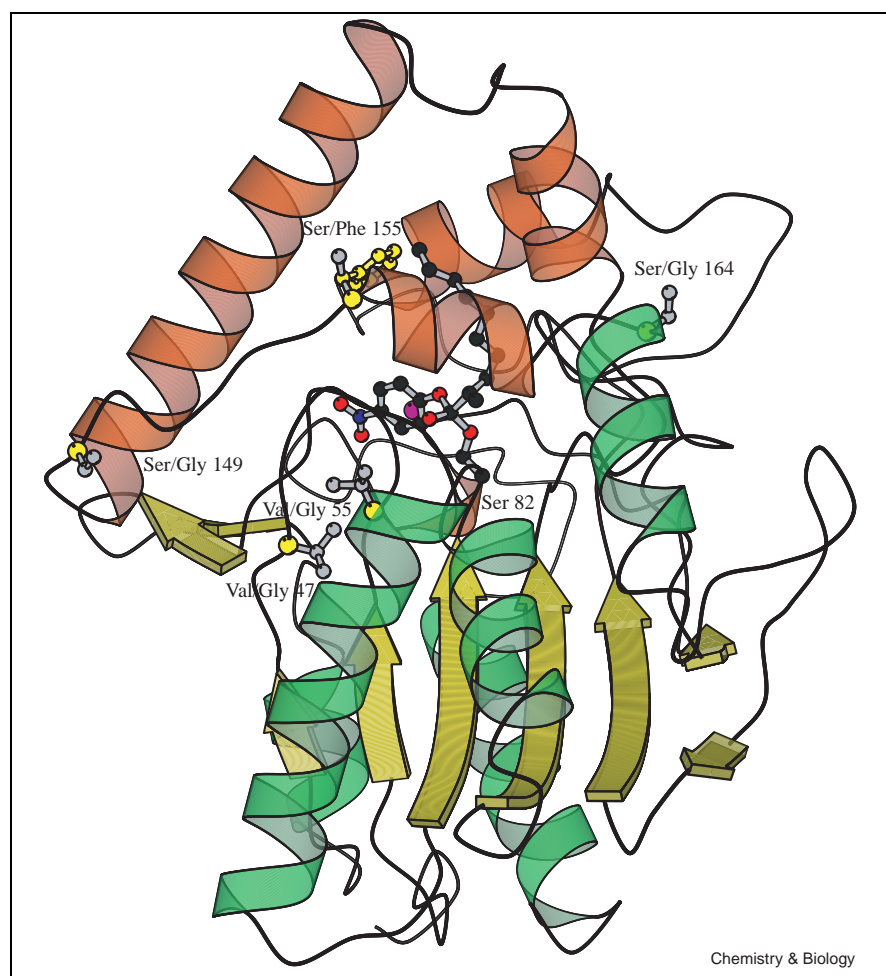
variant library, it is not to be expected that the specific amino acids introduced are in fact optimal. We therefore combined ep-PCR-based random mutagenesis with site-specific saturation mutagenesis to introduce all amino acids at those positions which had previously been identified as being important for enantioselectivity (generations 1–4) [10]. Saturation mutagenesis was then applied leading to the creation of libraries generally consisting of about 800 clones which were screened for enantioselectivity. No further improvements were found upon performing saturation mutagenesis at positions 149 (1. generation), 47 (3. generation) and 259 (4. generation). Assuming that all 19 substitutions had been generated, this result would indicate that the previously identified substitutions might already be optimal at these positions. However, the introduction of phenylalanine instead of a leucine previously introduced at position 155 (2. generation) raised the  $E$ -value from 4.4 for mutant S155L to 5.7 for mutant S155F (Figure 1). Subsequently, saturation mutagenesis at position 155 was performed with the 3. generation clone which already contained the amino acid substitutions S149G, S155L and V47G. Once again, the best performing mutant contained the substitution S155F leading to an increase in enantioselectivity from  $E = 9.7$  to  $E = 20.5$ . This result suggested that the substitution S155F plays an important role in improving enantioselectivity of PAL in the test reaction, leading us to introduce this particular substitution by site-directed mutagenesis into those variants which had been identified in the fourth and fifth generation. Indeed, the selectivity factor increased from  $E = 10.7$  to 14.7 and from  $E = 12.0$  to 20.1, respectively (Figure 1). Finally, introduction of phenylalanine at position 155 in the wild-type li-

pase by site-directed mutagenesis led to a variant with  $E = 9.5$ .

Our results clearly demonstrate that the combination of ep-PCR-based random mutagenesis and saturation mutagenesis is a viable approach to increase the diversity of a variant library without making its size too large for complete enantioselective sampling. This is even more important if screening has to be performed rather than selection. The usefulness of combining mutagenesis methods has recently been demonstrated by Cherry et al. [24] and Miyazaki and Arnold [25] in other applications, namely in the improvement of the oxidative stability of a fungal peroxidase and of the thermostability of a psychrophilic protease, respectively. However, any meaningful application of saturation mutagenesis requires the prior identification of amino acid positions with relevance to the property under investigation. The fact that not all amino acids could be introduced at every single position by ep-PCR means that an unknown number of mutants potentially capable of high enantioselectivity cannot be generated with the method used. However, this is not at all detrimental to the approach in general, because the solution to the problem of finding a highly enantioselective enzyme for a given reaction is not unique, i.e. a family of mutant enzymes is likely to be accessible displaying the desired features.

#### Effects of combination of substitutions

The 4. generation variant containing the substitution S155F ( $E = 14.7$ ) exhibited a lower enantioselectivity as compared to the 3. generation variant containing the



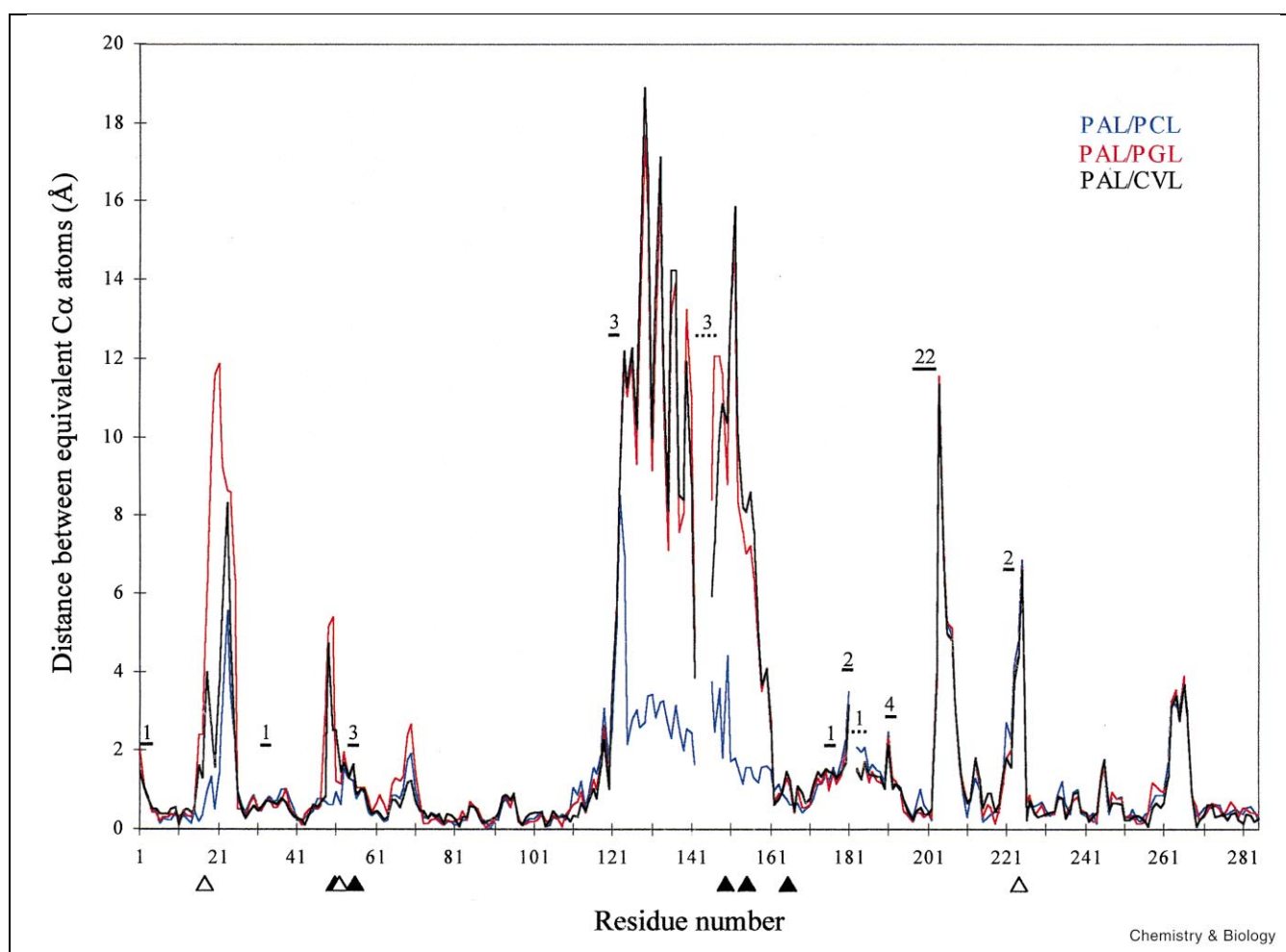
**Figure 2.** Structural model of the best performing *P. aeruginosa* lipase variant 94G12. Amino acid substitutions V47G, V55G, S149G, S155F and S164G identified in this variant were modeled into the structure of PAL [20] and are shown in a 'ball and stick' representation. The wild-type residues are in gray and the corresponding mutated residue in yellow. The substrate 2-methyldecanoic acid *p*-nitrophenyl ester was modeled into the active site using the coordinates of a covalently bound inhibitor. The substrate is shown in 'ball and stick' representation with its carbon, oxygen and nitrogen atoms in black, red and blue, respectively. The calcium ion present in the structure is shown in purple.

same substitutions ( $E = 20.5$ ), indicating that the combination of substitutions S155F and F259L which is present in the 4. generation has a negative effect on enantioselectivity (Figure 1). Further structural change by the substitution L110R identified in the 5. generation seems to compensate for this negative effect, raising the  $E$ -value to 20.1. At present, there are no obvious explanations available for these interesting effects (see discussion below).

Another illuminating aspect of our results pertains to the substitution S149G introduced in the 1. generation. This particular substitution increases the  $E$ -value from 1.1 to 2.1 with respect to the wild-type, but it does in fact have a negative effect when combined with the substitution S155F since it reduces the  $E$ -value of the corresponding variant S155F from 9.5 to 5.7. The S149G substitution is present in all generations derived from this variant and it may exert a negative effect in variants of other generations as well. We therefore decided to eliminate substitution S149G from the best performing mutants, hoping to find a further improvement in enantioselectivity.

These observations highlight a general problem of directed evolution executed by stepwise accumulation of randomly generated mutations. Initially beneficial mutations created in early generations may exert a negative effect when combined with other mutations generated in later generations. A possible solution to this problem could be saturation mutagenesis at the relevant position, in the present case at position 149. A more general and more efficient strategy could be the recombination of the evolved variants with (i) the parental wild-type gene, a process termed back-crossing known from traditional plant and animal breeding, or (ii) with few positive variants obtained from one generation [26]. A number of methods for *in vitro* [27–30] and *in vivo* recombination [24,31] have recently been developed.

Nevertheless, at this stage we decided to see how far one can go using point mutations only. Indeed, another round of ep-PCR on the variant which gave an  $E$ -value of 20.5 and on the variant containing only the substitution S155F led to the identification of the mutants 94G12 and 30E7



**Figure 3.** Comparison of the of C $\alpha$  atom positions for lipases from *P. aeruginosa*, *Pseudomonas cepacia*, *P. glumae* and *C. viscosum*. Differences between C $\alpha$  atom positions in the structures of PAL and PCL (blue) in the open conformation and PGL (red) and CVL (black) in the closed conformation are given in Å. Insertions and deletions in PAL are indicated above the relevant amino acid positions by solid and dotted lines, respectively. The number of inserted or deleted amino acids is indicated. The amino acid numbering corresponds to the PAL sequence. Loop regions involved in the transition from the closed to the open conformation are located between amino acids 16–25, 45–51 and 122–156. Other major deviations between C $\alpha$  atom position in the structures of PAL and the other lipases are caused by insertions or deletions. Solid arrow heads represent the position of amino acid substitutions identified in variant 94G12, open arrow heads those found in variant 30E7.

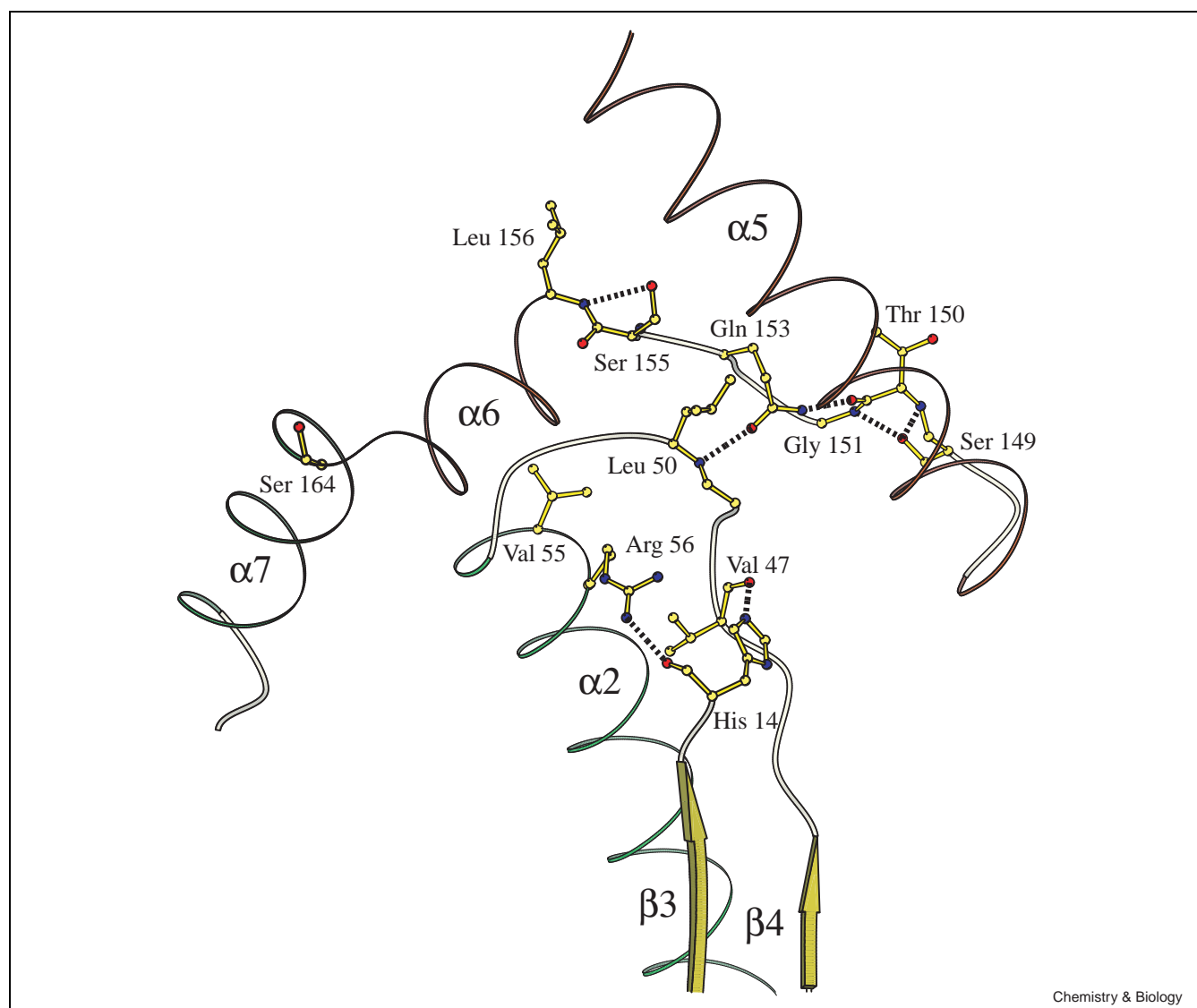
with even better enantioselectivities of  $E = 25.8$  (corresponding to  $ee = 91\%$  at 18% conversion) and  $E = 10.1$  (corresponding to  $ee = 79\%$  at 18% conversion), respectively (Figure 1).

Although PAL was evolved towards the hydrolysis of the *p*-nitrophenyl ester of 2-methyldecanoic acid, the enzyme showed similar enantioselectivity in reactions of the ethyl ester. For example, the mutant in the 4. generation displaying an  $E$ -value of 11.3 catalyzes the hydrolysis of the analogous ethyl ester with essentially the same enantioselectivity. This result indicates that *p*-nitrophenyl esters whose hydrolysis can easily be detected by spectrophotometric methods may well be used to evolve enzymes

which enantioselectively hydrolyze the corresponding chiral carboxylic acid alkyl esters.

#### Structural consequences of mutations

In the variant with the highest enantioselectivity (94G12), five substitutions have occurred (S149G, S155F, V47G, V55G, S164G), four of which involve the introduction of a glycine. This remarkable accumulation of glycine residues may increase the conformational flexibility of PAL. Flexibility has been identified as a possible factor determining the substrate selectivity of *Geotrichum candidum* lipase I [32,33]. Likewise, several conserved glycine residues in *Rhizomucor miehei* lipase were also shown to be important for the flexibility of this enzyme [34,35]. Inter-



**Figure 4.** Hydrogen bonding network around residues mutated in the 94G12 variant. Stabilizing interactions between the mutated residues Val47, Val55, Ser149, Ser155 and Ser164, and their nearby residues are indicated. Hydrogen bonds are shown in dashed lines.

estingly, the flexibility of *R. miehei* lipase is restricted to a few surface-exposed loops while the core of the enzyme is markedly rigid as deduced from several different molecular dynamics simulations on this lipase [34,36–39]. A comparable structural feature has been observed for different *Pseudomonas* lipases [40,41].

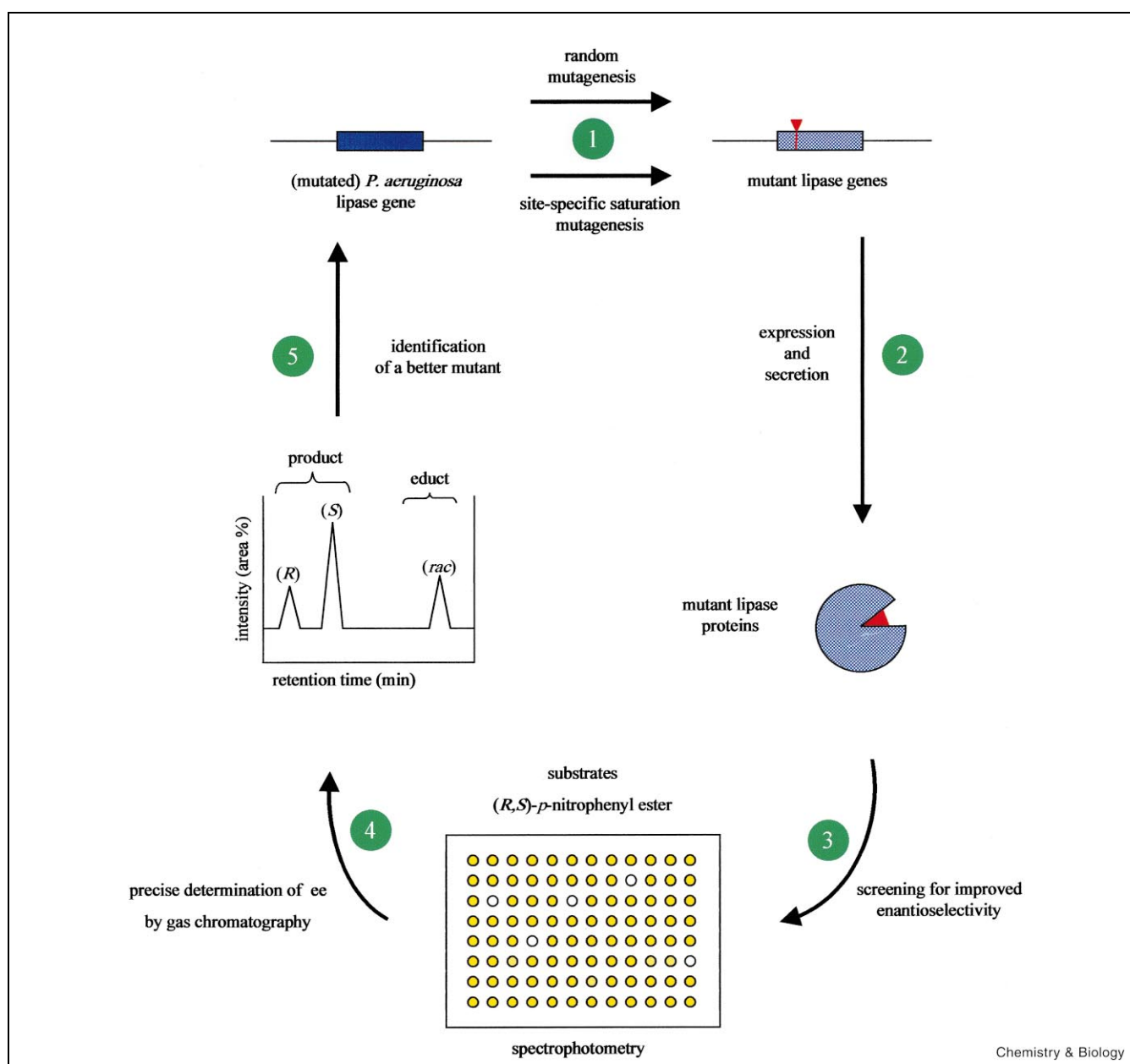
Recently, the 3D structure of PAL was solved in complex with a covalently bound inhibitor [20], which now allows us to locate the positions of the substitutions (see Figure 2) and to attempt to rationalize the effects of the mutations.

Almost all substitutions are located directly or in close

vicinity to loops which are involved in the enzyme's transition from the closed to the open conformation as shown in Figure 3 [19,20]. Surprisingly, all these substitutions are far from the stereocenter of the substrate, thus excluding a direct spatial effect on the enantioselectivity of the reaction.

The main chain atoms of Val47 and Val55 are directly or indirectly involved in a hydrogen bonding network which anchors His14 to helix  $\alpha 2$  and to the loop between strand  $\beta 4$  and helix  $\alpha 2$  (Figure 4). His14 is part of the loop containing the oxyanion residue Met16, therefore substitutions for glycines of Val47 and Val55 may not only increase the





**Figure 5.** Strategy for directed evolution of an enantioselective biocatalyst. Starting from the wild-type lipase gene *lipA*, mutations were introduced randomly by error-prone PCR or site-specific saturation mutagenesis (1). The resulting libraries were expressed in a lipase-deficient strain (2) and screened by a spectrophotometric assay towards 2-methyldecanoic acid *p*-nitrophenyl ester for clones expressing variant lipases with increased enantioselectivity (3). Variants with improved enantioselectivity were further analyzed by chiral gas chromatography (4). The gene of a better variant was chosen to parent the next generation (5).

flexibility of the region between strand  $\beta 4$  and helix  $\alpha 2$  but also affect the stability of the catalytically essential oxy-anion hole loop.

Ser149, Ser155 and Ser164 are located in the region between helices  $\alpha 5$  and  $\alpha 7$ . Their substitution for glycine may affect the secondary structure of the region following

the helix  $\alpha 5$ , not only because of an increased flexibility, but also because of the loss of hydrogen bonds between the side chains of S149 and Ser164, and nearby residues. Ultimately, this may even result in a somewhat different relative orientation of  $\alpha 5$  to  $\alpha 7$  helices. As this region contains residues which line part of the enzyme's substrate binding site that binds the acyl chain, a change in position



of these helices could easily affect the enzymes substrate binding properties. In particular, repositioning of the Leu162 side chain could have a large influence on the enantioselectivity of the enzyme as its side chain would directly interact with the methyl group of the (*S*)-substrate. Furthermore, other residues in the acyl binding pocket (Leu50, Thr158, Leu159 and Leu62) have van der Waals interactions with the side chain of Met16, one of the two amino acids which build up the oxyanion hole. Therefore, a variation of the position of the side chains of these residues might affect the oxyanion hole and thus the enantio-preference of the enzyme. This may also explain the variation in enantioselectivity upon introduction of the S155F substitution which would bring residue Phe155 at van der Waals distance to the acyl group of the substrate. Alternatively, the S155F substitution might directly influence the position of Met16 side chain and in that way induce a change in substrate preference.

## Significance

The results described here demonstrate for the first time that directed evolution can be used to evolve an enzyme displaying increased enantioselectivity over a significant range starting from  $E=1.1$  for the wild-type enzyme to  $E=25.8$  for the best mutant. The enzyme used in this model study is the lipase from *P. aeruginosa* (PAL) which catalyzes the hydrolysis of a chiral ester in the model reaction. We have shown that a successful directed evolution strategy based solely on point mutations can be developed which involves the initial localization of residues important for enantioselectivity of a model reaction by random mutagenesis using ep-PCR followed by the introduction of the optimal residue by saturation mutagenesis (Figure 5).

Sequencing studies led to the identification of those amino acid substitutions which are responsible for increased enantioselectivity. The elucidation of the 3D structure of wild-type PAL also allowed for initial structural conclusions. Furthermore, a molecular modeling analysis revealed that the flexibility of certain loop structures in PAL is of prime importance for enantioselectivity. We expect that the approach we have described will be viable for a variety of biotechnological applications. Moreover, studies of this type extend our knowledge of the structural basis of stereoselectivity in enzyme-catalyzed reactions. At present, we are pursuing a more extended exploration of PAL sequence space by (i) including recombinative methods for directed evolution, (ii) developing and applying more efficient screening methods for enantioselectivity, activity and stability, (iii) trying to invert the direction of enantioselectivity of PAL by evolving variants with high enantioselectivity towards the (*R*)- instead of the (*S*)-substrate and (iv) attempting to solve the 3D structure of selected mutant PALs in order to shed more light on the structural origin of enantioselectivity.

## Materials and methods

### Restriction of DNA

Restriction enzymes were purchased from MBI Fermentas (Germany) and NEB (Germany) and were used according to the manufacturer's instructions. DNA restrictions with two enzymes were performed in TAS buffer (33 mM Tris-acetate pH 7.9, 65 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol in 0.01 M sodium acetate pH 5.2, 0.4 mM spermidine).

### Expression of lipase genes

Lipase was expressed in *P. aeruginosa* PABST7.1, a lipase-deficient strain containing a chromosomally located copy of the T7-RNA polymerase under control of the *lacUV5/lacI<sup>q</sup>* element, allowing the induction of expression by addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG) [18]. Mutated lipase genes were expressed from plasmid pUCPL6A which were derived from pUCPKS [42] containing the lipase operon under control of the T7-promoter.

### Random mutagenesis

A 1046 bp *Bam*HI/*Ap*al fragment from plasmid pUCPL6A containing the complete lipase gene *lipA* was cloned into pBluescript II KS (Stratagene) giving plasmid pMut5 which served as the template DNA for error-prone PCR using primers LipA (5'-GCG-CAA-TTA-ACC-CTC-ACT-AAA-GGG-AAC-AAA-3') and LipB (5'-GCG-TAA-TAC-GAC-TCA-CTA-TAG-GGC-GAA-3') located downstream and upstream, respectively, of the *Bam*HI/*Ap*al fragment. Error-prone PCR was performed on a RoboCycler Gradient 40 (Stratagene, The Netherlands) under the following conditions: a total volume of 50  $\mu$ l of 67 mM Tris-HCl pH 8.8, 16.6 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6.1 mM MgCl<sub>2</sub>, 6.7 mM EDTA, 0.2 mM dNTPs, 10 mM  $\beta$ -mercaptoethanol, 10% (v/v) DMSO, 0.15  $\mu$ M each of primer LipA and LipB, contained 1 ng of template DNA and 2 U Goldstar Taq-polymerase (Eurogentec, Belgium). Ten parallel samples overlaid with 70  $\mu$ l paraffin were amplified using the following cycling protocol: 1  $\times$  (2 min 98°C), 25  $\times$  (1 min 94°C; 2 min 64°C; 1 min 72°C), 1  $\times$  (7 min 72°C). This protocol resulted in an average mutation frequency of 1–2 base substitutions per 1000 bp. PCR products were purified with the NucleoSpin kit (Macherey and Nagel, Germany) and restricted with *Bam*HI and *Ap*al. Fragments were separated by gel electrophoresis in a 0.8% agarose gel, the appropriate band was excised and purified with the NucleoSpin Extract kit (Macherey and Nagel).

### Ligation and transformation

Restricted PCR products were ligated to pUCPL6A previously hydrolyzed with the same restriction enzymes using T4 DNA ligase (Fermentas, Germany) by mixing 5 pmol each of insert and vector in a 10  $\mu$ l volume and incubation for 2 h at room temperature. In order to avoid cloning of the wild-type lipase gene caused by incomplete restriction of pUCPL6A and religation, the *Bam*HI/*Ap*al fragment from pUCPL6A was substituted with a *Bam*HI/*Ap*al fragment from plasmid pKS $\Omega$ XB containing the *Xho*I/*Bam*HI  $\Omega$ -cassette fragment from plasmid pHRP316 [43]. Ligation mixtures were diluted 1:10 in TMF buffer and used to transform *Escherichia coli* JM 109 cells. Competent cells were prepared by the method of Hanahan [44]. Plasmid DNA from all transformants was purified using the InViSorp Spin Plasmid Mini-kit (InViTtek, Germany) and used to transform *P. aeruginosa* PABST7.1 made competent by the method of Irani and Rowe [45]. Clones expressing active lipase protein were identified by replicating colonies from transformation plates on tributyrin agar plates where they formed clear halos. Tributyrin plates were prepared as follows: 0.75 g of Gum Arabic (Sigma, Germany) was dissolved in 7.5 ml of sterile A. dest. and mixed with 7.5 ml of tributyrin (Sigma). This mixture was added to 500 ml of LB agar and emulsified with a Ultraturrax T25 (Ika Labortechnik, Germany) for 1 min at 24 000 U/min prior to pouring the plates.

### Screening

Each colony of *P. aeruginosa* PABST7.1/pUCPL6A was picked with a

sterile toothpick and resuspended in a well of a 96-deep-well microtiter plate filled with 1 ml of 2×LB (20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl) containing 200 µg/ml carbenicillin and 50 µg/ml tetracycline. Cells were grown by shaking the plates overnight at 30°C. Lipase expression was induced by addition of 0.4 mM IPTG and further shaking at 30°C for 4–24 h. Cells were separated from the culture supernatant by centrifugation of the plates at 5000×g for 15 min. An aliquot of 50 µl was taken from each well and pipetted into two wells of a second microtiter plate each containing 10 µl 100 mM Tris–HCl pH 8.0, 30 µl A. dest. and 10 µl of the (*R*)- or (*S*)-enantiomer, respectively, of 2-methyldecanoic acid *p*-nitrophenyl ester in 10% DMF. Plates were shaken for 10 s and the reactions monitored at 410 nm for 6–10 min at 30°C using a Spectra-max-8 channel photometer (Molecular Devices, USA). Apparent *E*-values were determined from the ratio of the slopes of the linear part of the curves obtained for the (*R*)- and (*S*)-enantiomers [10]. Those clones showing an improved enantioselectivity as compared to the 'parent' of the corresponding generation were further analyzed by reacting a racemic substrate mixture with culture supernatant and subsequent analysis of the reaction products by chiral gas chromatography. The genes encoding lipases with improved enantioselectivity were sequenced (Eurogentec, Belgium). For this, the *Bam*HI/*Ap*al fragments from pUCPL6A were cloned into pBluescript II KS giving pMut5 which also served as the template for the next round of error-prone PCR.

#### Site-specific mutagenesis

The 'megaprimer' PCR method of Barlettino et al. [46] was used to introduce the substitution S155F into wild-type and mutant lipases. The first PCR reaction was performed as follows: A 50 µl volume of 75 mM Tris–HCl pH 9.0, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (w/v) Tween 20, 1.5 mM MgCl<sub>2</sub>, 10% (v/v) DMSO, 0.2 mM dNTPs, 0.2 µM each of the mutagenic primer (5'-GGT-ACC-CAG-AAT-TTT-CTG-GGC-TCG-CTG-3') and the primer LipA contained 1 ng of template DNA (pMut5) and 2 U of Goldstar Taq-polymerase (Eurogentec, Belgium). The cycling protocol was as follows: 1×(2 min 98°C), 30×(1 min 94°C; 2 min 64°C; 1 min 72°C), 1×(7 min 72°C). After separation from the template DNA by agarose gel electrophoresis, the PCR products were purified with the NucleoSpin Extract kit (Macherey and Nagel) and served as megaprimers in the second PCR which was performed similar to the first reaction with the following changes: in addition to primer LipA and the megaprimer, LipB was added as a third primer at a concentration of 0.2 µM. Plasmid pMut6 which served as the template was derived from pMut5 by restriction with *Kpn*I/*A*flIII, making ends blunt with T4 DNA polymerase (Fermentas, Germany) and religation. PCR products were handled as described for random mutagenesis.

#### Saturation mutagenesis

Saturation mutagenesis was performed as described for site-directed mutagenesis [46] with a mutagenic primer in which the codon under investigation had been randomized by mixing equal amounts of nucleoside phosphoramidates during synthesis. Primers were obtained from Eurogentec (Belgium).

#### Cell growth

Lipase protein was produced in larger amounts by growing *P. aeruginosa* PABST7.1/pUCPL6A in 10 ml of 2×LB medium containing 200 µg/ml carbenicillin and 50 µg/ml tetracycline in a 100 ml Erlenmeyer flask after inoculation with a single colony. Cells were grown overnight at 30°C, lipase gene expression was induced by addition of 0.4 mM IPTG and cells were further grown for 24 h.

#### Enzyme purification

Wild-type and variant 94G12 lipase protein were purified as follows: cultures were centrifuged for 30 min at 20 000 U/min in an SS34 rotor (Sorvall-DuPont, Germany). The culture supernatant was filter-sterilized (pore diameter: 0.45 µm; Schleicher and Schüll, Germany) and concentrated by a factor of 15 with a Centriprep-30 device (Millipore, Germany).

One ml of the concentrated culture supernatant was mixed with 1 ml 10 mM Tris–HCl pH 8.0, 100 mM NaCl and loaded onto a Fractogel EMD Bio SEC-chromatography column (length: 500 mm, inner diameter: 15 mm; Merck, Germany) at room temperature using a Shimadzu SPD-6A control unit. Proteins were eluted at 1 ml/min using the same buffer. The first fraction was collected after 25 min, the last fraction after 70 min with a Gilson M233XL fraction collector. Elution with a fraction volume of 1.5 ml was monitored at 210 nm. Fractions containing the highest lipase activity (usually 15–20 fractions) were pooled and loaded onto an Uno-Q1 column (Bio-Rad, Germany), pre-equilibrated with buffer A (20 mM Tris–HCl pH 8.0, 100 mM NaCl) and connected to an FPLC unit (Pharmacia, Sweden). Proteins were eluted at 0.5 ml/min with the following NaCl gradient: 0–7 min with buffer A, 8–17 min from 100 mM to 400 mM NaCl in buffer A, 18–27 min from 400 mM to 1 M NaCl in buffer A, 28–32 min 1 M NaCl, 33–37 min from 1 M to 2 M NaCl in buffer A. Fractions of 1.0 ml volume were assayed for lipase activity and protein purity was determined by SDS–PAGE [47].

#### Determination of lipase activity

Lipase activity was determined with *p*-nitrophenylpalmitate as the substrate according to the method of Winkler and Stuckmann [48].

#### Heat inactivation of lipase

Lipase preparations were incubated at 60°C for the indicated time periods and subsequently stored in an ice/water bath until determination of lipase activity.

#### Modeling studies

For docking of the substrate into mutant enzymes, the atomic coordinates of the refined structure of the wild-type PAL in complex with *R*<sub>C</sub>-(S<sub>P</sub>)-1,2-dioctylcarbamoylglycero-3-*O*-*p*-nitrophenyl octylphosphonate were used [20]. The docking of 2-methyldecanoic acid *p*-nitrophenyl ester was manually done using the program O [49]. The substrate was built using the program QUANTA and regularized using the CHARMM force field [50]. The charges on substrate atoms were calculated using the Gasteiger method as implemented in QUANTA. The acyl chain of the substrate was docked in the enzyme active site cleft at the same position and with the same conformation of the corresponding chain of the phosphonate inhibitor present in the crystal structure. The *p*-nitrophenyl ester was positioned in the alcohol binding pocket of the PAL active site cleft, keeping polar residues of the substrate approximately at the same position as found in the inhibitor complex X-ray structure. Mutations in the structure were manually made using the program O [49], choosing the best rotamer to avoid steric clashes. The structures were subsequently subjected to energy minimization, using a conjugate gradient routine implemented in CNS [51], in order to tidy up unacceptable close contacts and poor stereochemistry. Extensive energy minimization was applied until convergence was reached, leading to a model with good stereochemical quality and no residues outside the allowed regions in the Ramachandran plot other than the nucleophile, Ser82, as found in other members of the α/β hydrolase fold family [20,52].

#### References

- Sheldon, R.A. (1993). *Chirotechnology: Industrial Synthesis of Optically Active Compounds*. Dekker, New York.
- Ahuja, S. (1997). *Chiral Separations: Applications and Technology*. ACS, Washington, DC.
- Collins, A.N., Sheldrake, G.N. & Crosby, J. (1992). *Chirality in Industry: The Commercial Manufacture and Applications of Optically Active Compounds*. Wiley, Chichester.
- Collins, A.N., Sheldrake, G.N. & Crosby, J. (1997). *Chirality in Industry II: Developments in the Commercial Manufacture and Applications of Optically Active Compounds*. Wiley, Chichester.
- Jacobsen, E.N., Pfaltz, A. & Yamamoto, H. (1999). *Comprehensive Asymmetric Catalysis*. Vol. I–III, Springer, Berlin.
- Faber, K. (1997). *Biotransformations in Organic Chemistry*. 3rd edn., Springer, Berlin.

7. Arnold, F.H. & Volkov, A.A. (1999). Directed evolution of biocatalysts. *Curr. Opin. Chem. Biol.* **3**, 54–59.
8. Zhao, H., Moore, J.C., Volkov, A.A. & Arnold, F.H. (1999). Methods for optimizing industrial enzymes by directed evolution. In *Manual of Industrial Microbiology and Biotechnology*. (Demain, A.L. & Davies, J.E., eds.), 2nd edn., pp. 597–604, ASM, Washington, DC.
9. Kuchner, O. & Arnold, F.H. (1997). Directed evolution of enzyme catalysts. *Trends Biotechnol.* **15**, 523–530.
10. Reetz, M.T., Zonta, A., Schimossek, K., Liebeton, K. & Jaeger, K.-E. (1997). Creation of enantioselective biocatalysts for organic chemistry by in vitro evolution. *Angew. Chem. Int. Ed. Engl.* **36**, 2830–2832.
11. Reetz, M.T., Becker, M.H., Kühling, K.M. & Holzwarth, A. (1998). Time-resolved IR-thermographic detection and screening of enantioselectivity in catalytic reactions. *Angew. Chem. Int. Ed.* **37**, 2647–2650.
12. Reetz, M.T., Becker, M.H., Klein, H.-W. & Stöckigt, D.A. (1999). Method for high-throughput screening of enantioselective catalysts. *Angew. Chem. Int. Ed.* **38**, 1758–1761.
13. Janes, L.E., Löwendahl, A.C. & Kazlauskas, R.J. (1998). Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases. *Chem. Eur. J.* **4**, 2324–2331.
14. Guo, J., Wu, J., Siuzdak, G. & Finn, M.G. (1999). Measurement of enantiomeric excess by kinetic resolution and mass spectrometry. *Angew. Chem. Int. Ed.* **38**, 1755–1758.
15. Kazlauskas, R.J. & Bornscheuer, U.T. (1998). Biotransformations with Lipases. In *Biotransformations I*. (Kelly, D.R., ed.), 2nd edn., pp. 37–191, Wiley-VCH, Weinheim.
16. Schmid, R.D. & Verger, R. (1998). Lipases: interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed.* **37**, 1608–1633.
17. Jaeger, K.-E. & Reetz, M.T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.* **16**, 396–403.
18. Jaeger, K.-E., et al., & Reetz, M.T. (1997). Bacterial lipases for biotechnological applications. *J. Mol. Catal. B Enzym.* **3**, 3–12.
19. Jaeger, K.-E., Dijkstra, B.W. & Reetz, M.T. (1999). Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu. Rev. Microbiol.* **53**, 315–351.
20. Nardini, M., Lang, D.A., Liebeton, K., Jaeger, K.-E. & Dijkstra, B.W. (2000). Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation: the prototype for family I.1 of bacterial lipases. *J. Biol. Chem.*, in press.
21. Leung, D.W., Chen, E. & Goeddel, D.V. (1989). A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique (Philadelphia)* **1**, 11–15.
22. Moore, J.C. & Arnold, F.H. (1996). Directed evolution of a *para*-nitrobenzyl esterase for aqueous-organic solvents. *Nat. Biotechnol.* **14**, 458–467.
23. Eckert, K.A. & Kunkel, T.A. (1991). DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl.* **1**, 17–24.
24. Cherry, J.R. et al. (1999). Directed evolution of a fungal peroxidase. *Nat. Biotechnol.* **17**, 379–384.
25. Miyazaki, K. & Arnold, F.H. (1999). Exploring nonnatural evolutionary pathways by saturation mutagenesis: rapid improvement of protein function. *J. Mol. Evol.* **49**, 716–720.
26. Zhao, H. & Arnold, F.H. (1997). Functional and nonfunctional mutations distinguished by random recombination of homologous genes. *Proc. Natl. Acad. Sci. USA* **94**, 7997–8000.
27. Stemmer, W.P.C. (1994). Rapid evolution of a protein in vitro by DNA shuffling. *Nature* **370**, 389–391.
28. Zhao, H. & Arnold, F.H. (1997). Optimization of DNA shuffling for high fidelity recombination. *Nucleic Acids Res.* **25**, 1307–1308.
29. Zhao, H., Giver, L., Shao, Z., Affholter, J.A. & Arnold, F.H. (1998). Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.* **16**, 258–261.
30. Shao, Z., Zhao, H., Giver, L. & Arnold, F.H. (1998). Random-priming in vitro recombination: an effective tool for directed evolution. *Nucleic Acids Res.* **26**, 681–683.
31. Volkov, A.A., Shao, Z. & Arnold, F.H. (1999). Recombination and chimeragenesis by in vitro heteroduplex formation and in vivo repair. *Nucleic Acids Res.* **27**, e18.
32. Holmquist, M., Tessier, D.C. & Cygler, M. (1997). Identification of residues essential for differential fatty acyl specificity of *Geotrichum candidum* lipases I and II. *Biochemistry* **36**, 15019–15025.
33. Holmquist, M. (1998). Insights into the molecular basis for fatty acyl specificities of lipases from *Geotrichum candidum* and *Candida rugosa*. *Chem. Phys. Lipids* **93**, 57–65.
34. Peters, G.H., Toxvaerd, S., Olsen, O.H. & Svendsen, A. (1997). Computational studies of the activation of lipases and the effect of a hydrophobic environment. *Protein Eng.* **10**, 137–147.
35. Peters, G.H. & Bywater, R.P. (1999). Computational analysis of chain flexibility and fluctuations in *Rhizomucor miehei* lipase. *Protein Eng.* **12**, 747–754.
36. Norin, M., Olsen, O., Svendsen, A., Edholm, O. & Hult, K. (1993). Theoretical studies of *Rhizomucor miehei* lipase activation. *Protein Eng.* **6**, 855–863.
37. Norin, M., Haeflner, F., Achour, A., Norin, T. & Hult, K. (1994). Computer modeling of substrate binding to lipases from *Rhizomucor miehei*, *Humicola lanuginosa*, and *Candida rugosa*. *Protein Sci.* **3**, 1493–1503.
38. Peters, G.H., van Aalten, D.M.F., Edholm, O., Toxvaerd, S. & Bywater, J. (1996). Dynamics of proteins in different solvent systems: analysis of essential motion in lipases. *Biophys. J.* **71**, 2245–2255.
39. Peters, G.H., van Aalten, D.M.F., Svendsen, A. & Bywater, R. (1997). Essential dynamics of lipase binding sites: the effect of inhibitors of different chain length. *Protein Eng.* **10**, 149–158.
40. Schrag, J.D. et al. (1997). The open conformation of a *Pseudomonas* lipase. *Structure* **5**, 187–202.
41. Kim, K.K., Song, H.K., Shin, D.H., Hwang, K.Y. & Suh, S.W. (1997). The crystal structure of a triacylglycerol lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of a bound inhibitor. *Structure* **5**, 173–185.
42. Watson, A.A., Alm, R. & Mattick, J.S. (1996). Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* **172**, 163–164.
43. Parales, R.E. & Harwood, C.S. (1993). Construction and use of a new broad-host-range lacZ transcriptional fusion vector, pHRP309, for Gram<sup>−</sup> bacteria. *Gene* **133**, 23–30.
44. Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580.
45. Irani, V.R. & Rowe, J.J. (1997). Enhancement of transformation in *Pseudomonas aeruginosa* PAO1 by Mg<sup>2+</sup> and heat. *BioTechniques* **22**, 54–56.
46. Baretino, D., Feigenbutz, M., Valcarcel, R. & Stunnenberg, H.G. (1994). Improved method for PCR-mediated site-directed mutagenesis. *Nucleic Acids Res.* **22**, 541–542.
47. Laemmli, U.K. (1970). Cleavage of the structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
48. Winkler, U.K. & Stuckmann, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* **138**, 663–670.
49. Jones, T.A., Zou, J.Y., Cowan, S.W. & Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr. Sect. A Found. Crystallogr.* **A47**, 110–119.
50. QUANTA96 Molecular Simulation Inc., Burlington, MA.
51. Bruenger, A.T. et al. (1998). Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **D54**, 905–921.
52. Ollis, D.L. et al. (1992). The  $\alpha/\beta$  hydrolase fold. *Protein Eng.* **5**, 197–211.